

Phenotype changes inherited by crossing pyrethroid susceptible and resistant genotypes from the cattle tick *Rhipicephalus (Boophilus) microplus*

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Abstract Dialelic crosses and backcrosses of pyrethroid resistant (RR) and susceptible (SS) *Rhipicephalus (Boophilus) microplus* tick strains were carried out and the substitution (Phe-Ile) within the sodium channel gene was monitored in order to analyze the effects of the genotype on the pyrethroid resistance phenotype as measured by the larval packet test (LPT). Parental strains: susceptible (SS) and resistant (RR); dialelic crosses: RS ($\text{♂RR} \times \text{♀SS}$), and SR ($\text{♂SS} \times \text{♀RR}$); and backcrosses: RS × SS, RS × RR, SR × SS and SR × RR were infested on 280 kg calves. Resistance type (monogenic or polygenic) and effective dominance were determined based on the discriminant concentration (DC) for cipermethrine (0.5%), deltamethrine (0.09%) and flumethrine (0.01%). Allele specific PCR (AS-PCR) was used for genotyping, looking at a sodium channel mutation (Phe-Ile substitution). The mortality rates and allele frequency of susceptible and pyrethroid resistant reference strains were 0% mortality and 90% RR alleles for resistant strain, and 100% mortality and 0% RR alleles as measured by the larval packet test (LPT) and allele specific PCR (AS-PCR) respectively. Backcrossed strain SR × RR showed an effective dominance (D_{ML}) of 0.605 for cypermethrin, 0.639 for deltamethrin and 0.498 for flumethrin, while survival of backcrosses RS × SS, RS × RR and SR × SS showed a

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significant tendency to recessivity. Backcrossed strain SR × RR (69.4%) also showed a higher RR genotype frequency with regards to RS × SS (25.5%), RS × RR (36.7%) and SR × SS (32.0%), however, susceptible allele was inherited in general as an incomplete dominant trait. Monogenic inheritance hypothesis was tested and the results showed monogenic inheritance for cypermethrin and flumethrin ($P < 0.05$) but not for deltamethrin ($P > 0.05$). However, significant correlation was found between RR genotype and the survival rate for all three pyrethroids used ($P < 0.05$), suggesting that a single substitution on the sodium channel gene can be responsible for resistance to pyrethroids as a class, due to the high frequency for RR genotypes. Combination with different mutations or metabolic resistance mechanisms cannot be excluded.

Keywords Acaricides · Tick control · Kdr · Point mutations · Sodium channel

Introduction

The cattle tick *Rhipicephalus (Boophilus) microplus* is one of the most important ectoparasites affecting the cattle industry in tropical areas basically because of its haemato-phagous behaviour and pathogens transmission (Brossard and Wikel 2004). Tick control has relied on the use of acaricides such as: Organophosphorous (OP), synthetic pyrethroids (SP), amidinas (Am), and macrocyclic lactones (ML) (Aguilar and Rodríguez 2003; Rodríguez et al. 2005, 2010). Currently, multiple resistance to acaricides is the most common phenotype found in some areas in México (Rodríguez et al. 2006), and SP resistance is a worldwide problem due to its continued use as an insecticide in a wide range of plagues (Field and Williamson 2001).

Resistance to pyrethroids has been associated to the presence of point mutations on the sodium channel gene in several insects, also named “Knockdown resistance” (Kdr) (Reviewed by Guerrero and Pruet 2003). This type of resistance decreases the acaricidal effect of pyrethroids as a class for insects and ticks control (Soderlund and Knipple 2003; Dong 2007).

There are some evidences pointing out the role of enhanced metabolic detoxication in pyrethroid resistance mediated by esterases (Hernandez et al. 2002) in addition to the target site insensitivity mechanism (sodium channel mutation) (He et al. 1999; Miller et al. 1999; Jamroz et al. 2000; Guerrero et al. 2001; Hernandez et al. 2002). However, the most common mechanism of pyrethroid resistance in mexican field populations is strongly associated to the substitution Phe → Ile, located at the domine III segment 6 (III-S6) (Rosario-Cruz et al. 2005, 2009).

Research on acaricide resistance has been conducted in order to elucidate the inheritance mechanisms to several molecules used as acaricides. Stone and Youlton (1982), evaluated some Australian strains resistant to diazinon and cholpyriphos demonstrating an incomplete dominance of two complementary genes responsible of resistance. There is some evidence suggesting that OP resistance in Mexican strains is due to the participation of several genes (Harris et al. 1988).

Tapia et al. (2003), found that resistance to flumethrin is autosomically inherited and controlled by more than one gene. Li et al. (2005), established that Amitraz resistance is inherited in the cattle tick *R. microplus* as an incomplete recessive trait controlled by more than one gene responsible of amitraz resistance in a Brazilian strain analyzed. Recently, Li et al. (2008) carried out genetic studies on a Brazilian *R. microplus* tick strain resistant to

permethrin and found that permethrin resistance is inherited in an incomplete recessive manner.

Phenotypes and genotype changes have been analyzed in the progeny obtained from reciprocal crosses between pyrethroid susceptible and resistant genotypes (Aguilar et al. 2008). However, studies based on backcrosses of F1 with susceptible and resistant parental strains have shown to be useful in other insect models (Tabashnik 1991), therefore the aim of the present study was to analyze the changes on susceptibility produced by backcrossing heterozygous RS and SR *R. microplus* genotypes, with both parental SS and RR strains, based on the presence or absence of sodium channel mutation as measured by PCR. Susceptibility to pyrethroids was measured by the discriminant concentration (DC) in the LPT and genotype frequency by AS-PCR in order to elucidate how pyrethroid resistance is inherited based on the changes in susceptibility and monitoring the frequency of resistant genotypes.

Materials and methods

Backcrosses

Parental strains RR and SS previously described and the F1 progeny obtained by reciprocal crosses between resistant males and susceptible females ($RR\delta \times SS\varphi$) as well as susceptible males and resistant females ($SS\delta \times RR\varphi$) named RS and SR (first letter male and second letter female) respectively, were genotyped and tested by the LPT using a DC for each of the three pyrethroid molecules used in this experiment (Aguilar et al. 2008).

Both heterozygous strains, RS and its reciprocal cross SR, were backcrossed with parental SS and RR strains, briefly 250 mg of larvae from RS strain (approximately 5,000) were mixed up with both 250 mg of SS strain (named RS \times SS) and 250 mg of RR (named RS \times RR). Both strains were infested in separated calves. Strains SR \times SS and SR \times RR were obtained backcrossing SR strain with both parental strains SS and RR by the same procedure mentioned above (letter at first position belongs to a male and the second position to a female). Parental strains RR and SS as well as Backcrosses (RS \times SS, RS \times RR, SR \times SS and SR \times RR) were all infested on individual calves and kept in separated compartments. Engorged females from each backcrossed strain were collected from calves approximately 21 days after and placed on petri dishes (25 each) and incubated at 28°C and 85% Relative humidity (Cen et al. 1998). After oviposition, eggs were transferred into two glass vials (10 ml) with a cotton cap. Larval hatching occurred approximately 30 days after collection of engorged females. Live larvae of 7–14 days of age were used for bioassays (Kemp et al. 1998) and genotype determination. One vial was used for LPT bioassays and the other was frozen at –70°C until single larvae genomic DNA for AS-PCR assays was obtained.

Larval packet test

The modified bioassay of larval packet test (LPT) (Stone and Haydock 1962) was used to test pyrethroids susceptibility. Discriminant concentration for cypermethrin, deltamethrin and flumethrin (technical grade compounds) were: 0.5% (4.18×10^{-5} mM/cm²), 0.09% (6.22×10^{-6} mM/cm²) and 0.01% (6.85×10^{-7} mM/cm²) respectively. Briefly, acaricides dissolved in a mixture of trichloroethylene and olive oil (2:1 ratio) were used to treat filter papers that were then set for 2 h in a fume hood to allow trichloroethylene to

evaporate before being folded into packets using bulldog clips. Approximately 100 tick larvae from each backcrossed strain were added into the treated filter papers packets, which were then sealed with bulldog clips and placed in an incubator (28°C and 85% RH). Each concentration of the acaricide was replicated three times as well as the control (Olive oil and trichloroethylene). The treated larvae were exposed 24 h to the acaricide and the numbers of live and dead larvae were counted to calculate the percentage of larval mortality.

Genotyping

Genomic DNA was extracted from individual tick larvae and an allele specific PCR was performed as previously described by Guerrero et al. (2001), in order to look at the presence of the Phe-Ile substitution within the S6 transmembrane segment from domain III of the sodium channel (He et al. 1999) and then calculate the allele and genotype frequency.

For each tick population, 50 larvae were tested. Each tested larvae had three possibilities to be genotyped, RR is the homozygous resistant (possess both mutated sodium channel alleles), RS and SR are the heterozygous resistant-susceptible (possess one wild type and one mutated sodium channel) and SS is the homozygous susceptible (possess the two wild-type alleles). Also the resistant allele (R) frequency was calculated and defined as the percentage of substituted sodium channel allele in the total number of alleles assayed (2 alleles per individual).

Statistical analysis of data

Larval mortality for pyrethroids was assess by the LPT using the DC for cypermethrin, deltamethrin and flumethrin on parental strains (SS and RR) and backcrosses (RS × SS, RS × RR, SR × SS and SR × RR). Mortality records obtained by testing tick susceptibility at the DC by the LPT for each pyrethroid, were transformed (arc sin $\sqrt{}$ /percentage of mortality transformation) in order to obtain a homogenous variance with a normal distribution. Tukey test was used in order to find statistically significant differences between strains (SAS).

Effective dominance was calculated by using the formula described by Bourguet et al. (2000), for cypermethrin, deltamethrin and flumethrin, as it follows:

$$D_{ML} = (ML_{RS} - ML_{SS}) / (ML_{RR} - ML_{SS})$$

D_{ML} = varies between 0 and 1 (0 = survival is recessive; 1 = survival is dominant).

Effective dominance determines the relative mortality level (ML) for a certain acaricide concentration. DC (twice as much the LC_{99.9} of susceptible reference strain) was used to discriminate susceptible from resistant strains by the LPT as well as D_{ML} calculations.

In order to test the one single gene hypothesis for pyrethroid resistance, results of mortality observed values from the progeny of backcrossed strains were compared to the expected values by using the Chi square (χ^2) test and the formulas described by Sokal and Rohlf (1981):

$$Y_x = 0.50 (W_{RS} + W_{RR})$$

where: W_{RS} = The RS mortality obtained under a defined concentration (F_1) and W_{RR} = The RR mortality obtained under a defined concentration.

$$\chi^2 = (F_1 - \hat{p}n)^2 / \hat{p}\hat{q}n$$

where: F_1 = the observed number of dead larvae at a defined concentration; \hat{p} = The proportion of dead larvae; $\hat{q}=1-\hat{p}$; and n = is the total number of backcrossed larvae exposed to a defined concentration.

The association between RR genotype frequency and survival rate at the DC for cypermethrin, deltamethrin and flumethrin on both parental (RR and SS) and backcrossed strains (RS × SS, RS × RR, SR × SS and SR × RR), was analyzed by Pearson's correlation coefficient, calculated by using the statistical software SPSS ver. 13.

Results

The genotype frequency, effective dominance and dose-mortality responses using a DC for each pyrethroid used in the study and the effective dominance are shown in Table 1. Briefly, larvae from the susceptible (SS genotype) and resistant (RR genotype) parental strains, were included in the study, as well as the F1 from reciprocal crosses RS (RR♂ × SS♀) and SR (SS♂ × RR♀), were backcrossed with both SS and RR strains and RS × SS, RS × RR, SR × SS and SR × RR strains were obtained. Both parental and backcrossed strains (RS × SS, RS × RR, SR × SS and SR × RR) were tested for pyrethroid susceptibility by LPT bioassay and genotype frequency determined by allele specific PCR in order to elucidate the hypothesis of single or multiple genes involved with pyrethroid resistance as well as the inheritance of the sodium channel mutation as a recessive or dominant trait.

Significant differences in mortality rates were found between backcrossed strains (RS = resistant male and susceptible female) RS × SS and RS × RR when exposed to deltamethrin and flumethrin but no differences were notice when exposed to cypermethrin.

Mortality rates for the heterozygous strains RS and SR showed significant differences when backcrossed with the susceptible strain (SS) for cypermethrin and flumethrin but no differences were notice to deltamethrin, on the other hand the same heterozygous strains backcrossed with resistant strain (RR) showed differences in mortality rates for cypermethrin and deltamethrin but no differences when exposed to flumethrin.

Genotype RR frequencies for backcrossed strains RS × SS, RS × RR SR × SS and SR × RR, were 25.5, 36.7, 32.0 and 69.4%, respectively, as measured by allele specific PCR done on single larvae genomic DNA as a template.

Chi square analysis of dose response data as measured by the LPT bioassay showed significant differences (Table 2) for deltamethrin resistance, but not for cypermethrin and flumethrin, therefore deltamethrin resistance is probably due to more than a single gene, while cypermethrin and flumethrin most likely due to a single gene. Changes in phenotypes as measured by the LPT bioassay were consistent with the genotype frequency since a highly significant correlation $P < 0.05$ estimated by the determination coefficient (r^2) was found for all three pyretroids used: cypermethrin ($r^2 = 0.96$), deltamethrin ($r^2 = 0.95$) and flumethrin ($r^2 = 0.90$) (Fig. 1).

Discussion

Recent studies on the cattle tick *R. microplus* has been done in order to elucidate how resistance to amitraz and permethrin is inherited using the LPT as a diagnostic test to assess

Table 1 Larval mortality percentage from both parental (RR and SS) and backcrossed strains (RS \times SS, RS \times RR, SR \times SS and SR \times RR) as measured by using the LPT and DC for cypermethrin (0.5%), deltamethrin (0.09%) and flumethrin (0.01%), effective dominance (D_{ML}), RR genotype frequency for parental and backcrossed strains as measured by allele specific PCR

Strain genotypes	Cypermethrin mortality (%)	Deltamethrin mortality (%)	Flumethrin mortality (%)	Cypermethrin (D _{ML})	Deltamethrin (D _{ML})	Flumethrin (D _{ML})	RR genotype (%)
SS	100 ^a	100 ^a	100 ^a	0	0	0	0
RS × SS	79.48 ^b	81.58 ^b	80.45 ^b	0.205	0.184	0.195	25.53
RS × RR	69.25 ^{bc}	56.78 ^c	59.29 ^{cd}	0.307	0.432	0.407	36.73
SR × SS	63.45 ^c	78.65 ^b	62.37 ^c	0.365	0.213	0.376	32.00
SR × RR	39.46 ^d	36.07 ^d	50.18 ^d	0.605	0.639	0.498	69.38
R ^e	0 ^e	0 ^e	0 ^e	1	1	1	90

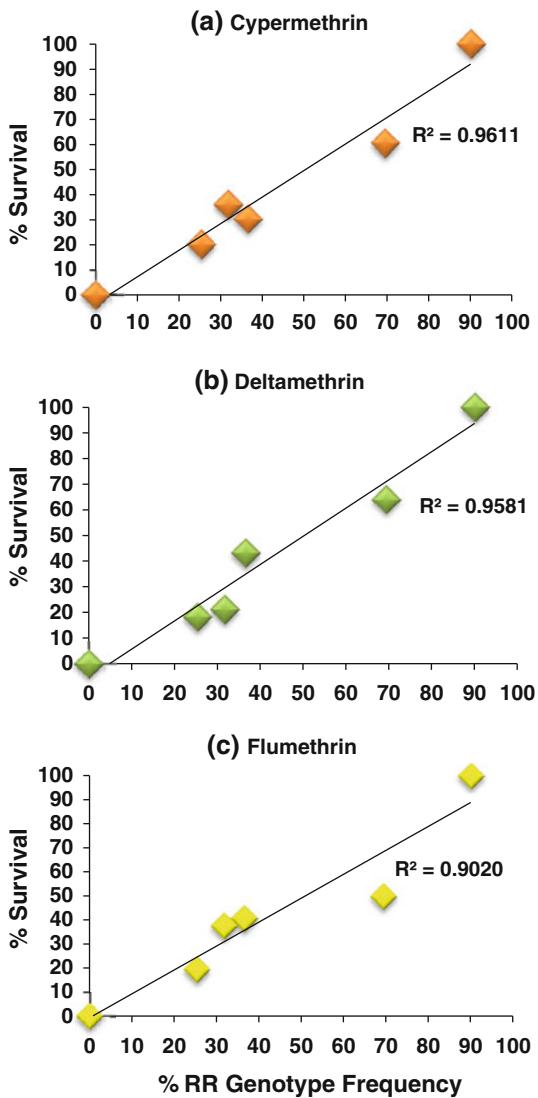
Statistical differences between rows and columns are denoted by different super index lowercase letters

Table 2 Observed values and Chi Square (χ^2) values calculated for testing the single gene hypothesis controlling resistance to cypermethrin, deltamethrin and flumethrin as measured by the LPT and the DC ($2 \times \text{CL}_{99.9}$) for both parental (RR and SS) and backcrossed strains (RS \times SS, RS \times RR, SR \times SS and SR \times RR)

ACARICIDE	DC (%)	TT(OV)	TD(OV)	TA(OV)	F1– $\hat{p}n$	$(F1 - \hat{p}n)^2$	\hat{p}	\hat{q}	$\hat{p}\hat{q}$	$\hat{p}\hat{q}n$	$\chi^2 = (F1 - \hat{p}n)^2 / \hat{p}\hat{q}n$
Cypermethrin	0.5	478	224	254	2.41	5.8	0.463	0.536	0.248	118.86	0.048
Deltamethrin	0.09	502	235	267	-41.14	1,692.4	0.550	0.449	0.247	124.24	13.62*
Flumethrin	0.01	572	316	256	20.88	435.9	0.515	0.484	0.249	142.85	3.05

* Significant difference; $F1$ observed number of dead larvae in a backcrossed strain at the DC; \hat{p} the proportion of dead larvae based on equation number 1; $\hat{q} = 1 - \hat{p}$; n total number of backcrosses exposed to the DC; W_{RS} RS mortality obtained at DC (F1). W_{RR} RR mortality obtained at DC; TT total number of larvae tested; TD total number of dead larvae; TA total number of alive larvae; OV observed value

Fig. 1 Correlation between RR genotype frequency and survival percentage using the DC ($2 \times LC_{99.99}$) for cypermethrin, deltamethrin and flumethrin to assess the pyrethroids dose–response from parental (RR and SS) and backcrossed strains (RS × SS, RS × RR, SR × SS and SR × RR) by the LPT at the DC



the offspring susceptibility (Li et al. 2005, 2008), however, genotypes have not been considered in these studies. Tapia et al. (2003) found that flumethrin resistance is controlled by more than one gene, since no significant differences were found by comparing the CL₅₀ from the reciprocal crosses RS and SR, no maternal effects and no sexual association was found in resistance to flumethrine. Those findings did not match with our studies when we analyze both the dose–response test to pyrethroids and monitoring of RR genotypes assessed by the allele specific PCR assay.

Based on the results of RS (δ RR × ♀SS) hybrid strain, resistance to flumethrin and deltamethrin is due to a single gene, since mortality rates for both acaricides were 100%, respectively, and effective dominance ($D_{ML} = 0$) for both acaricides were also 0.00, suggesting that the resistance for both flumethrin and deltamethrin is inherited in a

completely recessive mode. However, the hybrid SR (δ SS \times ♀RR) from a resistant female showed effective dominance values of 0.380, 0.319 and 0.258 for cipermethrin, deltamethrin and flumethrin, respectively; therefore, a maternal effect cannot be excluded, the resistance to pyrethroids seems to be inherited in a partially recessive mode when the female is resistant, probably due to more than one mechanism (Aguilar et al. 2008).

Similar results have been found by Mebrahtu et al. (1997) for *Aedes aegypti* in a study of permethrin resistance. Amitraz resistance has been also studied in the cattle tick *R. microplus* by Li et al. (2005). Similar findings were observed between reciprocal crosses RS and SR strains in term of the CL₅₀ (0.0089 and 0.0219% respectively) and the resistance index (3.7 and 9.1 respectively), suggesting a maternal effect on the SR strain derived from the resistant female.

Different formulae were used for the analysis of dominance, since the studies mentioned above were based on the CL₅₀, which was not calculated in this study due to the high pyrethroid resistance index of the resistant strain; therefore the study was based on the DC ($2 \times LC_{99.9}$) in the LPT bioassay which has been used in previous studies in correlation with the R allele frequency (Rosario-Cruz et al. 2005).

Miller et al. (1999) have demonstrated that the magnitude of pyrethroid resistance in *R. microplus* due to the substitution in the sodium channel is significantly higher than the esterase based metabolic detoxification mechanisms. These results have been confirmed by Rosario-Cruz et al. (2009) in Mexican field strains, no significant differences in esterase activity but highly significant correlation between the larval mortality rates and RR genotype frequency were found.

Effective dominance (D_{ML}) values, for all the strains used in this study were calculated according to the formulae described by Bourguet et al. (2000) using the DC results ($2 \times LC_{99.99}$). Similar values were expected in the offspring from the heterozygous strain RS when backcrossed with SS and RR compared to the SR backcrossed with the same parental tick strains; however, mortality results obtained from the LPT using the DC, showed significant differences ($P < 0.05$) between RS and SR offspring when backcrossed with SS and RR genotype strains. Frequencies of RR genotypes of backcrossed strains did not show statistically significant differences, except for SR \times RR ($P < 0.05$) showing an increased RR genotype frequency of 69.38%, (Table 1). These results clearly show a significant increase of the RR genotype frequency suggesting a possible maternal effect, since its reciprocal strain (RS) contained only, 36.73% of RR genotypes when backcrossed with the same RR genotype strain.

Regarding the effective dominance for backcrossed strains, the survival in SR \times RR strain was inherited as a partially dominant trait supporting the hypothesis of a possible maternal effect associated to the SR \times RR strain, while it was found to be partially recessive for RS \times SS, RS \times RR and SR \times SS, supporting the single gene trait on the sodium channel, responsible of pyrethroid resistance.

Based on the analysis of the effective dominance, we can conclude that pyrethroid resistance in backcrossed strains RS \times SS, RS \times RR and SR \times SS, is inherited as a partially recessive trait ($D_M =$ below 0.5), however, the effective dominance of the strain derived from a resistant female (SR \times RR), ($D_M =$ above 0.5) indicates that for this particular strain, pyrethroid resistance is inherited by a partially dominant trait, probably due to a maternal effect inherited by the offspring SR \times RR which was originally derived from the SR heterozygous strain (derived from a RR female).

These results are consistent with the RR genotype frequencies, since there were no differences among the RS \times SS, RS \times RR and SR \times SS genotype frequencies (25.53, 36.73 and 32.00% respectively), while significant differences were found in SR \times RR

strain (69.38%), confirming the possible influence of a maternal effect. However, high correlation between dose response and genotype frequencies analysis, demonstrate that above 93% ($r^2 = 0.93$ average) of the mortality response is due to the presence of a single point mutation on the sodium channel gene ($r^2 = 0.96$ for cypermethrin; $r^2 = 0.95$ for deltamethrin and $r^2 = 0.90$) indicating by the significant association between the dose-response variables, that the point mutation and therefore the resistance to the pyrethroids as a class, is inherited as a recessive trait ($P < 0.05$), however, the involvement of metabolic detoxification pathways or combination with different mutations on the sodium channel gene cannot be rejected.

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